

## Characterization of Newcastle Disease Virus Isolated During 1995-2009 From Suburbs of Karachi-Pakistan

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**Abstract.-** Newcastle disease (ND) which is caused by *Avian Paramyxovirus* type 1 is among the major concerns of poultry production worldwide. In developing countries especially where backyard poultry are not monitored for infection, ND remains a threat. The use of vaccines (live and inactivated) for the last 50 years in South Asia has not been able to control the infection. The poultry mortality rate worsened after the emergence of Avian Influenza in commercial poultry during early 1990's. The present study was conducted on 84 samples during 1995-2009, from commercial poultry flocks in the suburbs of Karachi to isolate and characterize strains of Newcastle disease virus circulating in the region. The samples were passaged in 9-10 day old chicken embryonated eggs. Haemagglutinating activity was observed in eighty samples which were cross checked using ND virus (NDV) polyclonal serum employed in haemagglutination inhibition assay and only forty seven were positive for NDV. The infective allantoic fluid was later used for pathotyping of isolates by employing biological characterization tools. Results demonstrated that the majority of isolates were either mesogenic or velogenic strains based on the mean death time and intra cerebral pathogenicity indices. Molecular identification was done using reverse transcription polymerase chain reaction (RT-PCR) performed using viral RNA extracted from infective allantoic fluid. Three sets of previously reported primers were employed and primer set BK1/BK2 was found to be the most sensitive. The results suggest that Newcastle disease remains endemic and requires similar attention to that given to highly pathogenic avian influenza.

**Key words:** Newcastle disease, avian paramyxovirus type I, haemagglutinating activity, RT-PCR.

### INTRODUCTION

Newcastle disease (ND), an infection caused by avian paramyxovirus type 1 (APMV-1), was a major threat for the developed world in early 60's and late 70's (Alexander, 1998). The disease remains a problem for the developing world. Newcastle disease virus (NDV) continues to trigger backyard poultry outbreaks in developed countries such as U.S.A. and UK because of the importation of exotic birds (Alexander *et al.*, 1999a; Pedersen *et al.*, 2004) and racing pigeons (Kaleta *et al.*, 1988), respectively.

The disease is highly contagious and can infect approximately 236 avian species (Alexander, 1998). It exists as a single serotype based on neutralizing antibody test and cross protective

analysis (Alexander *et al.*, 1999a,b). It has been endemic in this region for six decades and outbreaks are still reported in Pakistan (Afzal, 1992; Akhtar and Zahid, 1995; Arsha, 2000). Live vaccines developed by local producers using indigenous strains has contributed to controlling the spread of disease (Rehmani, 1996). However the lack of regulatory statues on importation of live birds and poultry vaccines, have limited the impact of control efforts. In practice, infection and periodic outbreaks are not necessarily prevented through vaccination (Khalafall *et al.*, 1992; Oncel *et al.*, 1997; Roy *et al.*, 2000; Yang *et al.*, 1997, 1999), therefore, emerging NDV strains are of concern and may overcome vaccination barriers (Panshin *et al.*, 2002).

Avian influenza virus (AIV) infection from significant strains (*i.e.* H5, H7, H9) in combination with NDV has been reported to be negatively impacting the poultry industry in Pakistan (Naeem and Siddiqui, 2006; Naeem *et al.*, 1999, 2007).

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The aim of this study was to evaluate the disease status in Pakistan that might assist local vaccine producers to develop new vaccines and strategies for the industry.

## MATERIALS AND METHODS

### Viruses

Tracheal tissue samples were collected from commercial poultry flocks and backyard poultry in the suburbs of Karachi during the period of 1995-2009. They were immediately immersed in liquid nitrogen and transported to the lab for virus isolation. A 0.1 mL of the inoculum was injected in the allantoic cavity of 9-10 day old chicken embryonated eggs and incubated at 37°C for 48-72 hrs. The embryos containing dead or dying embryos were checked for haemagglutination activity (HA), fluid harvested and stored at -80°C for future study.

Eighty-four tracheal tissue samples (list not shown) were collected during 1995-2009 from commercial poultry farms and backyard poultry in Karachi. The highest numbers of samples were collected during 2007-2009 because of the emergence of AIV (H5). The samples were propagated in 9-10 day old chicken embryonated eggs and 93% were positive for spot haemagglutination. The infective allantoic fluid was then inactivated with appropriate inactivating agent (0.1% formalin) prior to being utilized for HA or haemagglutination inhibition (HI) assay.

### HA and HI assays

HA was performed in 96-well microtitre plates. The infective allantoic fluid was diluted two fold with sterilized normal saline and haemagglutination observed using 1.0% chicken RBCs (OIE, 2009).

For confirmation of NDV, beta procedure (constant virus, diluted serum) of HI was adopted (Beard and Wilks, 1973). Polyclonal sera against ND and AI (H5, H7 and H9) served as positive controls in HI. For this purpose, the known ND antiserum was diluted two fold with sterilized normal saline. Then 4 HA units of the infective allantoic fluid was added in each dilution. After incubation for 30 minutes, 1.0% chicken RBCs were added and HI titre was determined.

### Pathotyping

Biological characterization of the NDV involves evaluating the virus for pathogenicity using the indices as outlines by Office International Epizootics (OIE) *i.e.* intracerebral pathogenicity index (ICPI) and mean death time (MDT). The assays were carried out as described earlier (Alexander, 1998). In brief ICPI indexes of less than 0.7, between 0.7 to 1.5 and greater than 1.5 classifies the virus as lentogenic, mesogenic and velogenic respectively. As for the MDT, less than 60 h, between 60-90 h and more than 90 h classify the virus as velogenic, mesogenic or lentogenic respectively (Hanson and Brandly, 1955).

### Molecular identification of NDV

Molecular identification of NDV and AIV was carried out using one-step RT-PCR kit from Promega, USA as per manufacturer's instructions. The composition of reaction mixture was as follows:

Reaction buffer	10 µl
dNTP's mixture (100mM each dNTP's)	1 µl
Forward primer (50 pmol/µl)	1 µl
Reverse primer (50 pmol/µl)	1 µl
AMV reverse transcriptase (5u / µl)	1 µl
MgSO <sub>4</sub>	2 µl
Tfi DNA polymerase (5u / µl)	1 µl
Template	10 µl
RNAse free water	23 µl

PCR tubes were vortexed and spun down before being placed in the thermocycler (Eppendorf Mastercycler, Germany). Positive controls were prepared using NDV (Lasota) and AIV (H5, H7, H9) vaccine samples from market, while the negative control was prepared using nuclease free water. Primers shown in Table I were used for detecting the NDV and AIV samples. Table II shows PCR temperature cycle conditions for different primers.

Electrophoresis was performed using 5 µL of the amplified product in 1.0 % (w/v) agarose gel in 1 X TAE buffer at 100 volts for 40 minutes. Followed by staining with ethidium bromide (1 ug/mL) for 30 minutes and destaining with distilled water for 10 minutes. The gels were than viewed in Chemi Doc (Bio Rad, USA) and analyzed

**Table I.- Sequence of primers.**

Primer	Sequences	Product size (bp)	Virus	Reference
BK1	5' GGG AGG CAT ACA ACA GGA CA 3'	238	NDV	Kianizadeh <i>et al.</i>
BK2	5' TGG TTG CAG CAA TGC TCT C 3'			(1999)
ONDV1a	5' TGA CTC TAT CCG TAG GAT ACA AGA GTC TG 3'	1349	NDV	Ballagi-Pordany <i>et al.</i>
ONDV4a	5' GAT CTA GGG TAT TAT TCC CAA GCC A 3'			(1996)
NDV1	5' CTT TGC TCA CCC CCC TTG G 3'	275	NDV	Jestin and Jestin
NDV2	5' CTT CCC AAC TGC CAC TGC 3'			(1991)
NPF	5' TGT ACG GAC TTG CTG TGG CC 3'	106	AIV	Chaharaein <i>et al.</i>
NPR	5' GAG ACT GAA GAC CTG GCT GTT 3'			(2009)

**Table II.- Profile of thermal cycler.**

Primer	Rt	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold
BK1	45°C/ 45 min	95°C/ 2 min	95°C/ 30 sec	55°C/ 30 sec	68°C/ 30 sec	68°C/ 7 min	4°C/ ∞
ONDV1a	45°C/ 45 min	95°C/ 2 min	95°C/ 30 sec	55°C/ 30 sec	68°C/ 60 sec	68°C/ 7 min	4°C/ ∞
ONDV4a				40 cycles			
NDV1	45°C/ 45 min	95°C/ 2 min	95°C/ 30 sec	53°C/ 30 sec	68°C/ 30 sec	68°C/ 7 min	4°C/ ∞
NDV2				40 cycles			
NPF	45°C/ 45 min	95°C/ 2 min	95°C/ 30 sec	58°C/ 30 sec	68°C/ 30 sec	68°C/ 7 min	4°C/ ∞
NPR				40 cycles			

via Quantity One software version 4.5. Five  $\mu$ L 100 bp DNA molecular weight marker (Fermentas, Germany) was electrophoresed subsequently to compare the base size.

## RESULTS AND DISCUSSION

Results show that among the 93% of HA positive samples only 55% were ND positive, out of which 83% had solely NDV infection whereas the rest 17% had mixed infection with one of the avian influenza serotypes. The results obtained from pathotyping grouped 95% of NDV isolates with ICPI index > 0.7 and MDT of 87% with <60 hrs, 7% between 60-90 hrs and the rest above 90 hrs. Moreover, a 238 bp product with BK1/BK2 (Fig. 1) and 106 bp product with NPF/NPR (Fig. 2) was obtained for NDV and AIV isolates analyzed. A summary of the molecular identification using different primer sets is summarized in Table III. The

primer set BK1/BK2 was found to be most sensitive in comparison to the other used in this study to identify the NDV. This is suggestive that a constant genome sequence monitoring is needed for ND to detect future variability worldwide (Khan *et al.*, 2010; Wise *et al.*, 2004; Kim *et al.*, 2006).

**Table III: Percentage of identification using published primer sets for Newcastle disease virus.**

Virus	Primers (percentage positive)			
	BK1/ BK2	ONDV 1a F/ ONDV 4a R	NDV 1 F/ NDV 2 R	NPF/ NPR
NDV	100%	0%	30%	0%
AIV	0	0	0	100%

The highest number were collected during 2008, the time when the region was experiencing outbreaks as a result of infection by highly pathogenic avian influenza (HPAI, H5N1) (Ayaz *et*

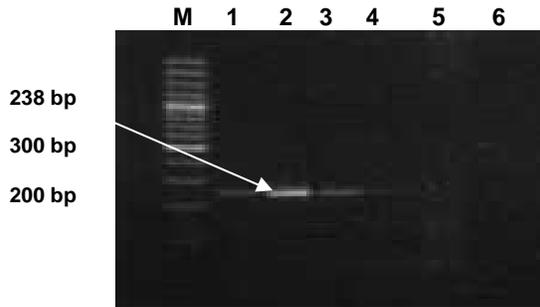


Fig. 1. A 238 bp fragment from Fusion gene of NDV with primer set BK1/BK2. M, marker (100 bp, Fermentas, Germany), Lane 1, positive control; Lane 6, negative control; Lanes 2-5, field samples.

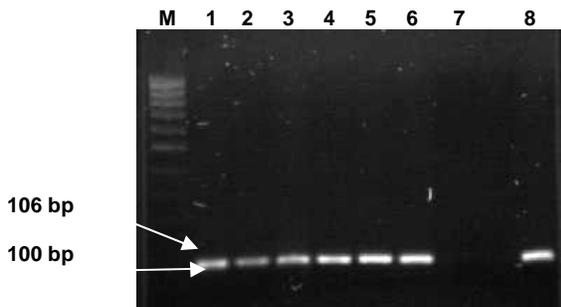


Fig. 2. A 106 bp fragment from nucleoprotein gene of AIV isolates with primer set NPF/NPR. M, marker (100 bp, Fermentas, Germany), Lane 8, positive control; Lane 7, negative control; Lanes 2-6, field samples.

*al.*, 2009). It was observed that NDV infection was occurring as an underlying infection along with AIV (Ayaz *et al.*, 2009). The pathotyping of the isolates as per OIE guidelines (OIE, 2009) classified the majority of samples as either mesogenic or velogenic in nature since the ICPI and MDT indices were greater than 0.7 and less than 90 hrs, respectively (Alexander, 2003).

Molecular identification of NDV, which was employed for the first time by Jestin and Jestin (Jestin and Jestin, 1991) using infective allantoic fluid and tissue, is now widely utilized with modifications for its detection (Creelan *et al.*, 2002; Gohm *et al.*, 2000; Kant *et al.*, 1997; Meulemans *et al.*, 2002) thereby eliminating the need for time consuming activities such as egg or tissue culture propagation of samples. ND is still a major

constraint for the development of the poultry industry in Pakistan and further epidemiologic studies are required to support the development of an effective and sustainable control program.

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## REFERENCES

- AFZAL, S., 1992. *Isolation and characterization of Newcastle disease virus strains in poultry*. Ph.D. thesis, Veterinary Microbiology, University of Agriculture, Faisalabad.
- AKHTAR, S. AND ZAHID, S., 1995. Risk indicators for Newcastle disease outbreaks in broiler flocks in Pakistan. *Prev. Vet. Med.*, **22**:61-69.
- ALEXANDER, D. J., 1998. Newcastle disease and other avian paramyxoviruses. In: *A laboratory manual for the isolation and identification of avian pathogens* (fourth edition) (eds. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson and W. M. Reed). American Association of Avian Pathologists, Kennet Square. pp. 156-163.
- ALEXANDER, D.J., 2003. Newcastle disease, Other Avian Paramyxoviruses, and Pneumovirus infections. In: *Diseases of poultry* (eleventh edition) (eds. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne), Iowa State Press, pp. 63-99.
- ALEXANDER, D.J., BANKS, J., COLLINS, M.S., MANVELL, R.J., FROST, K. N., SPEIDEL, E.C. AND ALDOUS, E.W., 1999a. Antigenic and genetic characterization of Newcastle disease viruses isolated in outbreaks in domestic fowl and turkeys in Great Britain during 1997. *Vet. Rec.*, **145**:417-421.
- ALEXANDER, D.J., MANVELL, R.J., BANKS, J., COLLINS, M.S., PARSONS, G., COX, B., FROST, K.M., SPEIDEL, E.C., ASHMAN, S. AND ALDOUS, E.W., 1999b. Experimental assessment of the pathogenicity of the Newcastle disease viruses from outbreaks in Great Britain in 1997 for chickens and turkeys, and the protection afforded by vaccination. *Avian Pathol.*, **28**:501-511.
- ARSHA, M., 2000. *Epidemiology and immuno-prophylaxis of Newcastle disease in poultry*. Ph.D. thesis, Veterinary Microbiology, University of Agriculture, Faisalabad.
- AYAZ, A., TASEER, A. K., BENAIZIR, K., YASIR, R., MUHAMMAD, A., SHAFQAT, F. R., NAZIR, A. L.

- AND SHAHANA. U.K., 2009. Molecular identification of agents causing respiratory infections in chickens from southern region of Pakistan from October 2007 to February 2008. *Int. J. Agric. Biol.*, **11**:325-328.
- BALLAGI-PORDANY, A., WEHMANN, E., HERCZEG, J., BELAK, S. AND LOMINCICZI, B., 1996. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. *Arch. Virol.*, **141**:243-261.
- BEARD, C. W. AND WILKS, W. J., 1973. A simple and rapid microtest procedure for determining Newcastle disease haemagglutination inhibition titers. In: *Proceedings of 77th Annual Meeting US. Animal Health Association*. pp. 596-600.
- CHAHARAEIN, B., OMAR, A. R., AINI, I., YUSOFF, K. AND HASSAN, S. S., 2009. Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription-polymerase chain reaction. *Microbiol. Res.*, **164**:174-179.
- CREELAN, J. L., GRAHAM, D. A. AND MCCULLOUGH, S. J., 2002. Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 from field cases using one-step reverse transcriptase-polymerase chain reaction. *Avian Pathol.*, **31**:493-499.
- GOHM, D. S., THUR, B. AND HOFFMAN, M.A., 2000. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. *Avian Pathol.*, **29**:143-152.
- HANSON, R. P. AND BRANDLY, C.A., 1955. Identification of vaccine strains of Newcastle disease virus. *Science*, **122**:156-157.
- JESTIN, V. AND JESTIN, A., 1991. Detection of Newcastle disease virus RNA in infected allantoic fluids by in vitro enzymatic amplification (PCR). *Arch. Virol.*, **118**:151-161.
- KALETA, E. F., ALEXANDER, D. J. AND RUSSELL, P. H., 1988. The first isolation of PMV-1 virus responsible for the current panzootic in pigeons. *Avian Pathol.*, **14**:553-557.
- KANT, A., KOCH, G., VAN ROOZELAAR, D.J., BALK, F. AND TER HUURNE, A., 1997. Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. *Avian Pathol.*, **26**:837-849.
- KHALAFALL, A. I., FADOL, M. A., HAMEID, O. A., HUSSEIN, Y. A. AND EL NUR, M., 1992. Pathogenic properties of Newcastle disease virus isolates in the Sudan. *Acta Vet. Hung.*, **40**:329-333.
- KHAN, T. A., RUE, C. A., REHMANI, S. F., AHMED, A., WASILENKO, J. L., MILLER, P. J. AND AFONSO, C.L., 2010. Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *J. Clin. Microbiol.*, **48** 1892-1894.
- KIANZADEH, M., IDERIS, A., SHAHRABADI, M. S., KARGAR, R., POURBAKSH, S.A., OMAR, A. R. AND YUSOFF, K., 1999. Biological and molecular characterization of newcastle disease virus isolated from Iran. *Arch. Razi Inst.*, **50** 151-161.
- KIM, L. M., AFONSO, C. L. AND SUAREZ, D. L., 2006. Effect of probe-site mismatches on detection of virulent Newcastle disease viruses using a fusion-gene real-time reverse transcription polymerase chain reaction test. *J. Vet. Diagn. Invest.*, **18**:519-528.
- MEULEMANS, G., VAN DEN BERG, T.P., DECAESSTECKER, M. AND BOSCHMANS, M., 2002. Evolution of pigeon Newcastle disease virus strain. *Avian Pathol.*, **31**:515-519.
- NAEEM, K. AND SIDDIQUE, N., 2006. Use of strategic vaccination for the control of avian influenza in Pakistan. *Develop. Biol.*, **124**:145-150.
- NAEEM, K., SIDDIQUE, N., AYAZ, M. AND JALALEE, M. A., 2007. Avian influenza in Pakistan: outbreaks of low- and high-pathogenicity avian influenza in Pakistan during 2003-2006. *Avian Dis.*, **51**:189-193.
- NAEEM, K., ULLAH, A., MANVELL, R. J. AND ALEXANDER, D. J., 1999. Avian influenza A subtype H9N2 in poultry in Pakistan. *Vet. Rec.*, **145**:560.
- O. I. E., 2009. Newcastle disease. In: *Manual of standards for diagnostic tests and vaccines* (ed. D.J. Alexander) IV, pp. 221-232. Office International Epizootics, Paris. Website: www.oie.int
- ONCEL, T., ALEXANDER, D. J., MANVELL, R. J. AND TURE, O., 1997. Characterization of Newcastle disease viruses isolated from chickens and pigeons in the South Marmara region of Turkey. *Avian Pathol.*, **26**:129-137.
- PANSHIN, A., SHIHMANter, E., WEISMAN, Y., ORVELL, C. AND LIPKIND, M., 2002. Antigenic heterogeneity among field isolates of Newcastle disease virus (NDV) in relation to the vaccine strain. 1. Studies on viruses isolated from wild birds in Israel. *Comp. Immunol. Microbiol. Infect. Dis.*, **25**:95-108.
- PEDERSEN, J. C., SENNE, D. A., WOOLCOCK, P. R., KINDE, H., KING, D. J., WISE, M. G., PANIGRAHY, B. AND SEAL, B. S., 2004. Phylogenetic relationships among virulent newcastle disease virus isolates from the 2002-2003 outbreak in California and other recent outbreaks in North America. *J. clin. Microbiol.*, **42**:2329-2334.
- REHMANI, S.F., 1996. Newcastle disease vaccination: A comparison of vaccines and routes of administration in Pakistan. *Prev. Vet. Med.*, **25**:241-248.
- ROY, P., VENUGOPALAN, A.T. AND MANVELL, R. J., 2000. Characterization of Newcastle disease viruses isolated from chickens and ducks in Tamilnadu. *Indian Vet. Res. Commun.*, **24**:135-142.
- WISE, M. G., SUAREZ, D. L., SEAL, B. S., PEDERSEN, J. C., SENNE, D. A., KING, D. J., KAPCZYNSKI, D. R. AND SPACKMAN, E., 2004. Development of a real-

- time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. *J. clin. Microbiol.*, **42**:329-338.
- YANG, C. Y., CHANG, P. C., HWANG, J. M. AND SHIEH, H. K., 1997. Nucleotide sequence and phylogenetic analysis of Newcastle disease virus isolates from recent outbreaks in Taiwan. *Avian Dis.*, **41**:365-373.
- YANG, C. Y., SHIEH, H. K., LIN, Y. L. AND CHANG, P. C., 1999. Newcastle disease virus isolated from recent outbreaks in Taiwan phylogenetically related to viruses (genotype VII) from recent outbreaks in Western Europe. *Avian Dis.*, **43**:125-130.

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